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(57) Abstract

The present invention provides improved methods for extracorporeal blood treatment, such as photochemotherapy, and related compositions are provided. The improved method involves introducing dendritic cells into the extracorporeal blood stream during agent treatment to further enhance the subject's immune system response to antigens present in the peripheral blood. The present invention further provides methods of identifying agents for use in the extracorporeal treatment of blood based on the ability of the agent to increase MHC expression.

METHODS FOR INDUCING IMMUNE RESPONSIVENESS IN A SUBJECT

Technical Field

The present invention relates to improved methods for inducing an immune response in a subject to cells or tissues, particularly tumor cells and/or T or B-cells that cause an autoimmune disorder. The present invention specifically provides methods and compositions for the extracorporeal treatment of blood and for administration of an extracorporeally treated blood mixture to a subject, to induce an immune response to cells or tissues that express a target antigen. In particular, the methods include using extracorporeal treatment agents, such as photochemotherapeutic agents, that increase MHC Class I peptide expression, and using/adding dendritic cells and/or other antigen presenting cells to the extracorporeal blood stream during treatment with such agents.

Background Art

Immune system responses may be classified as humoral or cell-mediated. A humoral response is mediated by B lymphocytes in the form of freely diffusible antibody molecules. A cell-mediated response is mediated by specifically reactive lymphocytes, such as T lymphocytes ("T cells"). T cells react with foreign antigens via surface receptors that are distinctive for each T cell clone. The T cell surface receptors generally are composed of two disulfide-linked protein chains having unique amino acid sequences (Edelson, R., *Annals of N.Y. Acad. of Sciences* 636:154-164 (1991)). The physical properties of these receptors confer specific binding capabilities and permit each of the several million clones of T cells in an individual to operate independently.

T cells function in the regulation of an immune response via recognition by the immune system of the T cell surface receptor. In the initiation of an immune response, the T cell receptor is capable of recognizing a particular antigen only when it is associated with a surface marker on an antigen presenting cell, such as a dendritic cell. These surface markers most commonly belong to a group of molecules known as the major histocompatibility complex (MHC). Binding of the T cell receptor to the

cleared from the subject's system by natural processes, but at an accelerated pace, presumably because of disruption of cell membrane integrity, alteration of the DNA within the cells, or related modifications.

More recently, methods and pharmaceutical compositions for specifically modifying an immune response to a specific antigen have been reported. These methods include treating an antigen-presenting cell to enhance expression by the cell of empty major histocompatibility complex molecules, followed by reacting the treated antigen presenting cell with an antigen extracorporeally in the presence of a photoactivatable agent and irradiation to form an antigen-associated antigen presenting cell. (See, e.g., PCT application number US93/11220, publication number WO 94/11016). None of the references and/or patents disclosed herein describes extracorporeal blood treatment methods that increase immune reactivity to specific target antigens and methods that can be used to augment existing photopheresis methods. Accordingly, there is still a need for improved methods and pharmaceutical compositions for inducing an immune response to a target antigen, for methods of preparing disease-associated antigen preparations which are specific for a subject and methods for augmenting existing photopheretic methods.

Summary of the Invention

The methods and compositions of the present invention are based on the identification that: 1) current agents used in photopheretic methods to induce an immune response to one or more target antigens are effective because they increase the level of MHC expression in the treated cell, 2) blood, containing disease effector cells, that is extracorporeally treated in known photopheretic methods results in the transport of disease associated antigens to the surface of the treated cells as weakly bound antigens to MHC molecules, 3) known photopheretic methods can be substantially improved by adding dendritic cells, or other antigen presenting cells, to the treated blood prior to re-infusion and 4) isolated and cultured dendritic cells can be used to boost a subject's immune system response in most context where an immune system response in desired.

increase the level of MHC Class I expression on the treated cells. Based on this observation the present invention provides methods for identifying agents for use in extracorporeal treatment methods in addition to the presently used photochemical agents. Further, this observation provides a means to optimize a treatment protocol by assaying for an increase in MHC Class I expression during agent treatments.

The methods and compositions of the present invention are used to treat a subject that has a disease that is mediated by or is conditioned upon the presence of circulating "disease effector" cells. Examples of disease effector cells include, but are not limited to, T cells, B cells, and/or infected white blood cells, such as virally or bacterially infected cells. Exemplary diseases that can be treated using the methods of the present invention include, but are not limited to, leukemia, lymphoma, autoimmune disease, graft versus host disease, and transplanted tissue rejection. In these conditions, an antigen that mediates the disease state (i.e., the "disease-associated antigen") is a peptide that is associated with (binds to) an MHC Class I site, an MHC Class II site or, to a heat shock protein that is involved in transporting peptides to/from MHC sites (i.e., a chaperone). Other conditions that can be treated using the present methods include condition in which a disease-associated antigen, such as a viral or bacterial peptide, is expressed on the surface of an infected white blood cell, usually in association with an MHC Class I or Class II molecule.

The methods and compositions of the present invention are useful for improving the effectiveness and specificity of therapeutic strategies that involve antigen presentation on any type of antigen presenting cell and in providing methods for inducing and augmenting an immune response to specific antigens. The invention is particularly useful for improving the efficacy of extracorporeal blood treatment methods, such as photopheresis, in subject populations for which photopheresis has proven of little or no value as a treatment modality, e.g., the 25% of subjects diagnosed with cutaneous T cell lymphoma for whom photopheresis has proven to be completely ineffective and the 50% of subjects in which the effect is transient and/or incomplete. The invention also provides a substantial cost savings by providing

Figure 2 shows the inhibition of tumor growth using dendritic cells (DAPC) alone.

Figure 3 shows the impact of 8-MOP/UVA treatment on MHC Class I expression.

Detailed Description of the Invention

A. General Description

The present invention provides improved methods for use in extracorporeal blood treatment for use in inducing an immune response in a subject. The improved methods use a synergistic combination of two therapeutic methods, extracorporeal treatment of blood with agents that increase MHC Class I expression (such as photopheresis) and dendritic or other antigen presenting cell-mediated immune therapy.

One embodiment of the present invention relates to the discovery that when combined, these two methods exert a synergistic therapeutic effect in treating a subject that is diagnosed as having a disease state that is mediated by circulating T cells, B cells or infected circulating cells, such as white blood cells infected by an infectious agent, such as, but not limited to, virus, bacteria, protozoa, etc.

B. Specific Descriptions

I) Extracorporeal Blood Treatment

According to one aspect of the present invention, an improved method for extracorporeal treatment of the blood of a diseased subject to induce an immune system response to one or more disease-associated antigens is provided.

In one embodiment, the methods of the invention comprise the steps of: 1) obtaining blood containing disease effector cells that express one or more disease associated antigens from the subject; 2) obtaining dendritic cells, or other antigen presenting cells, from the subject; 3) treating the blood containing the disease effector cells with an agent that increases MHC Class I expression (extracorporeal blood treatment); 4) introducing the dendritic cells, or other antigen presenting cells, into the treated blood mixture to form a therapeutic mixture; and (5) reinfusing or otherwise

pathological or disease state and 3) express peptides or proteins that can be used to distinguish them from other similar cells that are not associated with the pathological condition. For example, a non-circulating disease effector cell can be a solid tumor. Thus, disease effector cells (circulating and non-circulating) include, but are not limited to, malignant T cells, malignant B cells, T cells or B cells that mediate an autoimmune or transplanted tissue rejection response, virally or bacterially infected white blood cells or tissues that express viral or bacterial proteins/peptides and solid tumor cells. Preferably, the disease effector cells will express an antigen on the cell surface and will be T cells or B cells, more preferably T cells. The preferred disease effector cells are T cells belonging to a single clone. More preferably, the disease effector cells are T cells that are polyclonal or are tumor cells obtained from a solid tumor.

As described below, the agent treatment step of the methods of the present invention, for example photopheresis, damages the disease effector cells to the extent that the agent treated cells increase MHC Class I expression, and/or transport/release disease-associated antigens, but are not immediately lysed or killed. The transported/released peptides then are passed "baton-fashion" to the dendritic cell major histocompatibility complex molecules, either by entering empty MHC sites or by displacing peptides that are present in the added dendritic cell MHC Class I or Class II sites for presentation.

Thus, the methods of the invention are useful for treating diseases such as leukemias, lymphomas, solid tumors, metastatic tumors, autoimmune diseases, transplanted tissue rejection, and graft versus host disease. In addition, the methods of the present invention are useful for treating viral or bacterial conditions including HIV, malaria, etc. and other blood borne infections that are mediated by intracellular parasites or other factors, including, e.g., listeria, Epstein Barr virus, HTLV-1, herpes simplex, varicella, hepatitis A, B and C virus, protozoan, such as leishmania donovans. See, e.g., Goodman and Gilman's "The Pharmacological Basis of Therapeutics, W.A. Goodman Gilman *et al.*, Pergamon Press, N.Y., N.Y. (1990) for a description of protozoan infections which can be treated in accordance with the methods disclosed

cells per cc of blood. As can be readily appreciated, the blood used in the extracorporeal treatment may be removed over a course of several sessions. Alternatively the blood can be removed, treated and re-infused in a continuous process. For treating a solid tumor, as much of the tumor is removed for both therapeutic purposes as well as to provide a source of antigen for dendritic cell loading.

Agent Treatment of the Disease Effector Cells

After removal of blood from the subject, the blood containing disease effector cells, or purified disease effector cells, is subjected to extracorporeal treatment using a treatment agent. As used herein, "extracorporeal blood treatment" refers to the process in which the blood of a diseased subject is removed and is treated with an agent to form an agent-treated blood sample.

One observation of the present invention is that photochemical agents that have been used in photopheretic methods cause a previously known increase in MHC expression, particularly MHC Class I, on the treated cells. Accordingly, the agent used in the present method can be any agent that will act to increase MHC expression, particularly MHC Class I expression. For cells that do not express MHC proteins, any agent that leads to an increase in cellular proteolysis can be used. The treatment agent may further be an agent that has an affinity for an important component of blood cells or for a particular disease effector cell.

The agent used in the present method can be, but is not limited to, chemical agents and physical agents. For example, the agent may be a chemical compound that induces MHC expression and/or cellular proteolysis, such as a photoactivatable drug such as psoralen. Alternatively, the agent may be a physical treatment that the blood is subjected to. For example, UV light, heat shock and other environmental stresses have been shown to induce MHC Class I expression in other experimental contexts. As outlined below, a skilled artisan can readily identify agents for use in the present methods based on the ability of the agent to induce MHC expression on treated (disease effector) cells.

Exemplary photoactivatable chemical agents that can be used with the present methods include, but are not limited to, psoralens, porphyrins, pyrenes, phthalocyanine,

When using a photoactivatable agent, the agent-treated blood sample is further irradiated with ultraviolet or visible light during the agent treatment step, for example see U.S. Patent No. 5,462,733, issued to Edelson *et al.*, for a discussion of the irradiation conditions for activating photoactivatable agents such as psoralen compounds. Photopheresis procedures also are described in U.S. Patent Nos. 4,321,919; 4,398,906; 4,428,744; 4,464,166; and 5,147,289, all issued to Edelson *et al.*

The treatment of the blood or purified disease effector cells with the treatment agent can be done, as is known in the art, on a continuous stream of blood or in a batch wise manner. Continuous extracorporeal treatment can be divided into five stages: (1) blood collection; (2) centrifugation; (3) agent treatment; (4) cell pooling and (5) reinfusion. The choice of the agent treatment method used will be based primarily on the disorder being treated, the agent used and the facilities that are available.

During the agent treatment step, such as in the use of a photoactivatable agent, the agent can be present within or on the surface of the cells of the blood sample. This is typically accomplished by administering the agent to the subject prior to obtaining the blood for extracorporeal treatment or by injecting the agent directly into the extracorporeal blood stream when using a continuous stream treatment method.

In contrast to the literature that suggests that an underlying mechanism of photopheresis involves subtly modifying antigen presenting cells to enhance their ability to induce an immune system response, as shown in the Examples, treatment of the extracorporeal blood leads to an increase in MHC Class I expression and allows for an increase in the rate and extent that antigens are transported and bound (weakly) to surface MHC molecules. Such antigens then become available for presentation by dendritic or other antigen presenting cells, which are added to the blood sometime prior to reinfusion.

VI. Dendritic Cells Addition

The extracorporeal treatment methods of the present invention rely on the use of dendritic cells, or other antigen presenting cells, in combination with the

In one application, the dendritic cells can be activated *in vivo* prior to their removal from the subject. A variety of method can be used to activate dendritic cells *in vivo* prior to their removal. For example, activation can be accomplished by administering a sufficient dosage of GM-CSF to the subject prior to removing of the dendritic cells. As used herein a "sufficient dosage of GM-CSF" is the amount and frequency of administration of GM-CSF that is sufficient to increase the number and/or activation state of the dendritic cells in the subject. Exemplary dosages of GM-CSF for increasing the number and/or activation state of the subject's dendritic cells are provided in the Examples (see, "Isolation of Dendritic Cells from Human Blood").

In such a use, the steps of the present method comprise: 1) administering GM-CSF to the subject prior to removal of dendritic cells, wherein the dosage of GM-CSF is sufficient to increase the number and/or activation state of the dendritic cells in the subject; 2) obtaining blood containing disease effector cells that express one or more disease associated antigens from the subject; 3) obtaining and culturing, *in vitro*, dendritic cells, or other antigen presenting cells, from the subject; 4) treating the blood containing the disease effector cells with an agent that increases MHC Class I expression (extracorporeal blood treatment); 5) introducing the dendritic or other antigen presenting cells into the treated blood mixture to form a therapeutic mixture; and (6) reinfusing or otherwise introducing the therapeutic mixture into the diseased subject.

In general, the isolated dendritic cells can be introduced at any stage during the extracorporeal treatment process: (1) during the blood or tissue collection step; (2) prior to or after the disease effector cell isolation steps (i.e. centrifugation); (3) prior to or after the agent treatment step; and (4) prior to or after disease effector cell pooling. Thus, the dendritic cells can be introduced to the treated blood/tissue before, during, or after agent treatment. The advantage of introducing the dendritic cells during the blood collection stage is that a high dendritic cell concentration can be achieved by adding the dendritic cells directly to the blood collection bag. However, if centrifugation procedures are used to separate blood into plasma, white blood cell and red blood cell components it is not 100% efficient and some dendritic cells may enter

system response following reinfusion of the antigen-loaded dendritic cells to the subject.

Alternatively (or additionally), the dendritic or other antigen presenting cells can be added to the blood administration bag prior to reinfusing the agent treated disease effector cells. Methods for introducing the dendritic or other cells at any stage in the treatment process are based on conventional procedures and can be readily adapted for adding dendritic cells into a treated blood preparation. For example, at each stage of treatment process, conventional intravenous tubing connections provide access ports through which the dendritic cells can be injected into, for example, the blood collection bag, the centrifugation apparatus, the tubing located in the agent/irradiation treatment chamber and the blood re-infusion bag. Additional reagents, such as cytokines, also can be introduced via these same infusion ports.

In the above methods, the dendritic or other antigen presenting cells can be added directly or indirectly to the blood or tissue. As used herein, direct addition refers to adding the dendritic cells directly to the blood or tissues (before or after treatment) under condition in which there can be direct cell-to-cell contact between the added dendritic cells and the disease effector cells.

As used herein, indirect addition refers to adding dendritic cells to the blood or tissues (before or after treatment) such that the dendritic cells do not come into direct contact with the disease effector cells. For example, a filter membrane, dialysis membrane or other partitioning membrane can be placed in between the dendritic cells and the disease effector cells. Such a partition acts to allow the transfer of disease associated antigens to the dendritic cells but does not allow mixing of the cell types. The preferred partitions will have a pour size of no greater than about 6 microns since this is the approximate size of the small cell that would need to be prevented from passing through the partition. There is no lower limit, however, the pour size must be large enough to allow passage of antigens and other cytokines released from the treated disease effector cells. Alternatively, the treated cells can be remove from the treatment solution, for example by centrifugation, leaving released diseased associated antigens, and the dendritic cells can be added to the resulting cell free solution.

the precursors and culturing the precursors in the presence of a cytokine. Steinman *et al.* report that GM-CSF is an essential cytokine for dendritic cell culturing *in vitro*. Accordingly, Steinman *et al.* recommend administration of GM-CSF to a subject prior to sampling the subject's blood to obtain dendritic cell precursors for proliferation *in vitro*. Steinman *et al.* further report that the cultured, immature dendritic cells can be pulsed with antigen *in vitro* and will phagocytose the antigen and process it into a form which is presented on the dendritic cell surface, i.e., the Steinman dendritic cells must phagocytose the antigen for proper antigen presentation in Class II.

The Steinman method involves (a) providing a tissue source (e.g., blood, bone marrow) containing dendritic cell precursors; (b) treating the tissue source to increase its proportion of dendritic cell precursors to obtain a population of cells which is suitable for culture *in vitro* (e.g., by contacting the tissue source with GM-CSF); (c) culturing the tissue source on a substrate and in a culture media containing GM-CSF, or a biologically active derivative of GM-CSF, to obtain proliferating nonadherent cells and cell clusters; (d) subculturing the nonadherent cells and cell cultures to produce cell aggregates comprising proliferating dendritic cell precursors; and (e) serially subculturing the cell aggregates one or more times to enrich the proportion of dendritic cell precursors.

Mature dendritic cells are produced from the proliferating cell cultures by continuing to culture the dendritic cell precursors for a period of time sufficient to allow these cells to mature into mature dendritic cells. Mature dendritic cells are identified by cell markers such as, for example, high MHC Class II, 2A1 positive granules, and interdigitating cell (NLDC) antigen. In contrast to the Steinman *et al.* teachings, a preferred embodiment of the instant invention involves introducing mature dendritic cells into the treated blood to form disease-associated antigen-loaded dendritic cells. Thus, there is no requirements that the dendritic cells of the instant invention be in an immature state and capable of phagocytosis to present the disease-associated antigens of the invention.

Dendritic cells (or precursors) are cultured in the presence of GM-CSF, IL-4 and fibroblast growth factor at a concentration that is sufficient to promote the survival

(M1/42 anti-MHC Class I, ATCC number TIB 126); (2) those which bind to the MHC Class II antigen, B21-2 anti-MHC Class II, ATCC number TIB 229), (M5/114 anti-MHC Class II, ATCC number TIB 120); (3) those which bind to heat-stable antigen (M1/69 anti-heat stable antigen, HSA, ATCC number TIB 125); (4) 33D1 anti-dendritic cell antibodies, ATCC number TIB 227; (5) those which bind to the interdigitating cell antigen (NLDC 145 anti-interdigitating cell, Kraal, G., *et al.*, *J Exp Med* 163:981 (1986)); and (6) those which bind to antigens in granules in the perinuclear region of mature dendritic cells (monoclonal antibodies 2A1 and M342, Agger, R., *et al.*, *Int Rev Immunol* 6:89 (1990)). Additional antigens that are expressed by dendritic cells that can be used to identify mature dendritic cells include CD44 (identified with monoclonal antibody 2D2C), and CD11b (identified with monoclonal antibody M1/70). (See, e.g., Monoclonal Antibodies, New York, Plenum 1980, Ed. R. Kennett *et al.*, pp. 185-217 for a description of some of the monoclonal antibodies which are used to identify antigens which are expressed on mature dendritic cells). One skilled in the art will recognize that other antibodies may be used to characterize and identify mature dendritic cells and also to characterize and identify precursor dendritic cells and to distinguish these stages of dendritic cell growth.

Although mature dendritic cells are preferred for introduction into the treated blood, either during or following agent treatment, immature dendritic cells also can be pulsed with the disease-associated antigen preparations of the invention. Thus, contacting the mature or immature dendritic cells *in vitro* with the antigen preparations of the instant invention results in a composition containing antigen-loaded dendritic cells in which the antigen is presented on the surface of the dendritic cells. Although not intending to be bound to a particular theory, it is believed that the mature dendritic cells present antigen by loading the (released, disease-associated) antigen directly into the empty MHC sites or, alternatively, by exchanging the disease-associated antigens for peptides that already are present in the MHC sites of the mature dendritic cells. In contrast, the immature dendritic cells present antigen by the foregoing mechanism, as well as by phagocytosing released antigens, processing the released antigens into

al., *EMBO J* 5:943-949 (1986)). There is no requirement that a processing defect in the antigen presenting cells of the invention be complete, provided that the cells express an increased population of cell surface MHC Class I or Class II molecules which are devoid of endogenously processed peptides. Such cells are capable of inducing a primary CTL response when appropriately loaded with MHC Class I binding peptides.

The antigen presenting cells used in the present methods can also be antigen presenting cells that have been treated with antisense oligonucleotides to inactivate one or more genes responsible for proper antigen processing and presentation at the cell surface. This approach increases the number of empty Class I molecules on the antigen presenting cells, thereby making these cells more capable of binding antigenic peptides released from treated disease effector cells. Thus, for example, dendritic cells or other antigen presenting cells are incubated with antisense oligonucleotides under conditions to permit hybridization of the antisense oligonucleotide to the processing gene or mRNA (e.g., the human TAP-2 gene). The TAP genes encode proteins which are necessary for the transport of relevant cytoplasmic peptides to Class I molecules, prior to their joint transport to the cell surface. Therefore, inhibition of the formation of TAP proteins, diminishes filling of Class I molecules with peptides. This circumstance will, hence, increase the amount of surface "empty" Class I. Exemplary conditions and oligonucleotides for inactivating the TAP-2 gene in cultured RMA and EL4 cells or freshly isolated splenocytes are provided in Nair, S. *et al.*, *J Immunology* 156:1772-1780 (1996). In particular, S. Nair report that MHC Class I expression was decreased in approximately 30% of the cells which had been treated with the AS-1 or AS-2 antisense oligonucleotides. These oligonucleotides are complementary to two different regions of the TAP-2 mRNA and were synthesized as 25 nucleotide long phosphorothioate derivatives. (See, Nair, S. *et al.*, *ibid.* for sequence and storage information for these antisense oligonucleotides).

The following procedure, based upon that described by Nair, S. *et al.*, *ibid.*, is used for preparing a preferred antigen presenting cell for use in the present method. Briefly, antigen presenting cells (preferably in log phase) are washed in medium (e.g.,

1:1297-1302 (1995)) or an adhesion molecule (e.g., ICAM-1/CD54; ICA-3/CD50 (Young, J. *et al. J Exp Med* 183:7-11 (1996) and references cited therein); and (3) one or more of the MHC Class I molecules of the subject. Human B7-1 and B7-2 are described in Freedman, A.S., *et al.*, *J Immunol* 137:3260-3267 (1987), Freeman, G.J., *et al.*, *J Immunol* 143:2714-2722 (1989), Freeman, G.J., *et al.*, *Science* 262:909-911 (1993) and Azuma, M., *et al.*, *Nature* 366:76-79 (1993). Thus, an improved dendritic stock cell line can be prepared by introducing one or more cDNAs encoding a cytokine (preferably, GM-CSF) and an accessory molecule (preferably, a B7 and/or ICAM-1 molecule) into a processing defective cell line (preferably, a T2 cell line). (See, for example, Paglia, P., *et al.*, *J Exp Med* 183:317-322 (1996), for an exemplary procedure for transducing dendritic cells with the gene encoding GM-CSF in a mouse model system). The improved dendritic stock cell line can be prepared and maintained in accordance with standard procedures known in the art for introducing and expressing genetic material in mammalian (preferably, human) cells. Moreover, in the preferred embodiments, the dendritic stock cell line is used to prepare subject specific antigen presenting cells by, for example, introducing the cDNA encoding the subject's MHC Class I molecules into the stock cell line using standard genetic engineering procedures. Of course, such transformed antigen presenting cells are treated (e.g., gamma-irradiated) to prevent further cell division prior to administration to the subject. The amount and nature of irradiation that is sufficient to prevent further cell division is determined empirically by irradiating the cells with a preselected radiation source (e.g., gamma-irradiation, 8-MOP and ultraviolet irradiation, X-irradiation, 8-MOP and visible irradiation) over a range of intensities (e.g., 1000 to 3000 rads) for preselected time periods (e.g., 0.5 minutes to 24 hours) and observing whether any clones develop over a period of time, usually a one month period. The amount and nature of the irradiation is selected which is sufficient to prevent any clones from developing during this time period. Typically, 2000 rads of gamma-irradiation reportedly is sufficient to achieve this purpose. (See, e.g., Zitvogel, L., *et al.*, *J Exp Med* 183:87-97 (1996)).

In contrast to the above-described transformed cell lines which must be irradiated to prevent further cell division in vivo, cultured dendritic cells that are

WO 95/29698, WO 94/02156, WO 94/20127, WO 91/13632, WO 95/28479, WO 94/21287 and CA patent application 2,069,541.

VII. Reinfusion

After the dendritic or other antigen presenting cells are added to the treated blood (directly or indirectly) to form a therapeutic mixture, the mixture can be re-infused into the subject as a treated dendritic cell/treated cell mixture or can be further processed to obtain isolated, antigen-loaded dendritic cells. The former provides an efficient method that can further provide an additional source of disease cell antigen (i.e. the agent treated disease cell antigens), while the later removes potentially viable disease effector cells.

Re-isolating the dendritic cells prior to reinfusion provides a means for obtaining multiple re-infusion doses and a source of antigen-loaded dendritic cells that can be used for immunotherapy. Specifically, the peptide-loaded dendritic cells generated by contacting dendritic cells with agent treated blood, can be used as an immunogen by administering the cells to a subject in accordance with methods known in the art for eliciting an immune response. Preferably, the dendritic cells are injected into the same individual from whom the source cells were obtained. The injection site may be subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.m.), intradermal (i.d.), or intravenous (i.v.). Intravenous administration of the antigen-loaded dendritic cell is the preferred route of administration.

The number of antigen-loaded dendritic cells that are administered to the subject varies as a function of the antigen, the immune status of the subject the size of the subject and the disease that is treated. In a preferred embodiment, blood is used as the tissue source and preferably, the subject is first treated with cytokine to stimulate hematopoiesis. Following isolation and expansion of the dendritic precursor cells, the precursors are contacted with the antigen preparations made from agent treated disease effector cells and/or alternatively are stimulated by cytokines (e.g., GM-CSF) to maturity before introducing the antigen. The antigen-loaded dendritic cells are reintroduced to the subject in sufficient quantity to invoke an immune response. In general, between $1x10^6$ and $10x10^6$ dendritic cells constitute a single dose for injection

or eliminated tumor growth in an animal model without needing to contact the dendritic cell with a treated disease effector cell or disease associated antigen..

IX Antigen Containing Compositions

Although the prior art reports antigen-loaded dendritic cells for enhancing cellular immunity to a mixture of solid tumor antigens or to purified peptide antigens, methods for preparing disease-associated antigens by subjecting blood to agent treatment (such as photopheresis) and using the treated blood as an antigen source have not been described. Thus, according to another aspect of the invention, antigen compositions for enhancing an immune system response are disclosed. The antigen compositions have in common a plurality of disease-associated antigens that have been released from disease effector cells contained in blood by extracorporeal agent treatment as herein described. In addition, the compositions optionally contain a detectable amount of one or more protease or peptidase inhibitors. Exemplary protease or peptidase inhibitors and compositions containing the same include: (1) a mixture containing bestatin (30 uM), thiorphan (10 uM) and captopril (10 uM); (2) phenylmethylsulfonyl fluoride (a serine protease inhibitor); (3) n-ethylmaleimide and various nonselective peptidase inhibitors (e.g., EDTA, o-phenanthroline, bacitracin); (4) benzamidine (2x 10^2 mol/L); (5) a mixture of peptidase inhibitors including amastatin, captopril, phosphoramidon); (6) a mixture of peptidase inhibitors such as actinonin (6 uM), arphamenine B (6 uM), bestatin (10 uM), captopril (10 uM) and thirophan (0.3 uM); and (7) one or more of the protease inhibitors that are useful for treating HIV infection (e.g., ritonavir, saquinavir, indinovir).

The antigen compositions of the invention can be preserved at reduced temperatures (e.g., frozen to prevent bacterial growth) or alternatively, can be lyophilized for prolonged storage. Preferably, the antigen compositions are formulated to contain an amount of disease-associated antigens for mixing with a single dose of dendritic cells. In general, each dendritic cell contains approximately 100,000 MHC sites for binding to antigenic peptides. Accordingly, a single dose of dendritic cells is introduced into an excess of disease-associated antigens to drive the reaction to completion, i.e., to ensure that as many disease-associated antigens as possible are

at room temperature. In general, the acidification step involves subjecting the preparation of disease effector cells to a pH of between about pH 2 and pH 6 (preferably between pH2 and pH 4) for between about 0.5 to about 20 minutes. In the preferred embodiments, acidification involves subjecting the preparation to a pH of about 3.3 for about one minute. Thereafter, the cell preparation is neutralized in accordance with standard practice (e.g., by washing pelleted or flask-adherent cells with buffered tissue culture medium), and the eluted peptides preferably are further concentrated (e.g., by chromatography and/or lyophilization). Performing the acidification step under these conditions, results in release by the disease effector cells of their disease-associated antigens without immediately lysing the disease effector cells. Optionally, the acid-eluted antigen preparation is divided into aliquots, each aliquot containing an amount of antigen sufficient for mixing with a single dose of dendritic cells to enhance the immune system. Preferably, the aliquots are lyophilized to facilitate storage and shipping. Although not intending to be bound by a particular theory, it is believed that incubation of cells a pH 3.3 in citrate-phosphate buffer denatures Class I complexes, resulting in the release of beta2 microglobulin and previously Class I-bound peptides into the extracellular media (Storkus, W., *et al.* (*J Immunotherapy* 14:94-103 (1993)). Because the mild pH treatment does not immediately lyse the disease effector cells, the cells regenerate their Class I peptide complexes in culture, thereby providing a mechanism whereby multiple batches of disease-associated antigens can be harvested from the disease effector cells in culture.

In a particularly preferred embodiment, dendritic cells or other antigen presenting cells, are subjected to the above-described acid elution/neutralization protocol prior to contacting the cells with the disease-associated antigens. In this manner, the MHC molecules of the dendritic cells are emptied of their endogenous peptides prior to exposure to the disease-associated antigens, thereby increasing the number of empty MHC molecules available for association with the disease-associated antigens and rendering the acid-eluted dendritic or other antigen presenting cells more efficient antigen presenting cells, presumably, by providing an increased number of empty MHC sites into which the disease-associated antigens can be loaded. It is

specific T-cell-mediated immune response. More recently, methods have been reported for the isolation of precursor dendritic cells and their expansion *in vitro*. For example, PCT Application No. PCT/US93/03141 having publication Number WO 93/20185, entitled "Method for in vitro Proliferation of Dendritic cell Precursors and their use to produce Immunogens" (Steinman *et al.*, hereinafter WO 93/20185) describes methods for isolating dendritic cell precursors from human blood, expanding the isolated cell precursors *in vitro* in the presence of GM-CSF, and pulsing the expanded cell precursors with peptide antigen *in vitro* to obtain peptide-loaded dendritic cells that are suitable for inducting an immune system response. The following procedures for isolating and culturing dendritic cells/dendritic cell precursors from human peripheral blood are based upon the protocols for culturing such cells that are described in WO 94/02156 and WO 93/20185.

(A) Isolation and Culture of Dendritic Cell Precursors obtained from Human Blood

The procedure described herein is adapted from the isolation and culturing protocols provided in WO 93/20185 (Steinman *et al).*. Briefly, blood mononuclear cells are isolated by sedimentation in standard dense medium, such as Lymphoprep (Nycomed, Oslo). The isolated mononuclear cells are depleted of cells that are not dendritic cell progenitors. For example, these contaminants are coated with monoclonal antibodies to CD3 and HLA-DR antigens and depleted on petri dishes coated with affinity-purified, goat anti-mouse IgG ("panning"). Approximately 10^6 cells in one ml of culture medium are plated in 16 mm diameter plastic culture wells (Co-star, New York). The medium (e.g., RPMI-1640) is supplemented with typical growth nutrients (e.g., 50 uM 2-mercaptoethanol, 10 mM glutamine, 50 µg/ml gentamicin, 5% serum from cord blood without heat inactivation or 5% fetal calf serum (with inactivation)) and human recombinant GM-CSF (preferably 400 U/ml). Optionally, serum-free medium that is appropriate for mammalian cell culture can be used. Every second day thereafter and for a total of 16 days, the cultures are fed by removing 0.3 ml of the medium and replacing this with 0.5 ml of fresh medium supplemented with the cytokines. Preferably, the cells are cultured n the presence of

PCT/US91/01683, publication number WO 91/13632, entitled "Idiotypic Vaccination Against B Cell Lymphoma" ("Hohlen *et al.*") describes a protocol for isolating dendritic cells from spleen. The Hohlen *et al.* protocol is based upon the method previously reported by Steinman and Cohen, *J Exp Med* 139:380-397 (1974).

(B) Isolation of Dendritic Cells from Human Blood

The procedure described herein is adapted from the isolation and culturing protocols provided in WO 94/02156 (Engleman *et al.)*. Although dendritic cells are found in both lymphoid and nonlymphoid tissues, the most readily accessible source of dendritic cells in man is peripheral blood, which contains less than about 1 dendritic cell per 100 disease effector cells. To obtain a sufficient number of dendritic cells directly from blood without necessitating dendritic cell culture, a disease effector cell concentrate is prepared in accordance standard leukapheresis practice. In general, approximately two billion disease effector cells are collected during leukapheresis. Thus, assuming that the dendritic cells represent one percent of the total disease effector cell population collected by leukapheresis, approximately 20 million dendritic cells are present in the leukapheresis disease effector cell concentrate. As discussed below, this number of cells is sufficient to perform multiple treatments in accordance with the methods disclosed herein. In addition, further culture of these dendritic cells can be performed to increase further the total number of dendritic cells for therapy. For the *in vivo* priming of an immune system response, a highly purified dendritic cell population (of at least about 80%, preferably of at least about 90%) is recommended.

The number of dendritic cells present in blood and, hence, in a leukapheresis disease effector cell concentrate, can be increased by administering one or more agents which stimulate hematopoiesis prior to photopheresis or leukapheresis. Such agents include G-CSF, GM-CSF and may include other factors which promote hematopoiesis. The amount of hematopoietic agent to be administered is determined by monitoring the cell differential of subjects to whom the factor(s) are administered. Typically, dosages of cytokine agents, such as G-CSF and GM-CSF, are similar to the dosages of these agents that are administered to treat subjects recovering from treatment with cytotoxic agents. Preferably, GM-CSF or G-CSF is administered for 4 to 7 days at standard

Briefly, human dendritic cells can obtained from buffy coats using the following procedure. Peripheral blood mononuclear leukocytes (PBML) are isolated by Ficoll-Hypaque gradient centrifugation (Bouyam, *Scand J Clin Lab Invest* 21:21-29 (1968)). Blood dendritic cells optionally are further separated by, for example, the methods described in WO 94/02156. (See, in particular, WO 94/02156, Fig. 1, for an overview of the separation process). Briefly, PBML are separated into LD and HD fractions in a four-step discontinuous Percoll gradient (Pharmacia Uppsala, Sweden) (Markowicz and Engleman, *J Clin Invest* 85:955-961 (1990)). The HD fraction containing the dendritic cells is collected and cultured in culture media in Teflon vessels for 16-28 hours at 37°C. Thereafter, the cells are centrifuged over a Nycodenz/Nycopret discontinuous gradient (Nycomed Pharma, Oslo, Norway). The dendritic cells are contained entirely in the LD fraction and occupy approximately 30-40% of the total cell population. This partially purified dendritic cell population can be used for T cell priming and activation experiments *in vivo* or *in vitro*.

In the preferred embodiments, the dendritic cells are further purified for *in vivo* applications. Further purification of the dendritic cell population is achieved by performing a second round of Nycodenz/Nycoprep centrifugation and collecting the LD fraction obtained therefrom. The LD fraction contains approximately 80-90% dendritic cells. Alternatively, the LD fraction following the first Nycodenz/Nycoprep step is incubated with antibody-coated petri dishes to remove $CD3^+$, $CD14^+$, $CD16^+$, and $CD20^+$ cells to obtain a nonadherent cell population containing between approximately 80-90% dendritic cells. In general, these procedures produce a yield of $1\text{-}2.5\text{x}10^6$ cells from about 400-500 ml of whole blood.

Assessment of dendritic cell purity following enrichment is determined by staining with an anti-HLA-DR antibody (e.g., an anti-MHC Class II antibody such as CA141) which is conjugated to a detectable reagent (e.g., fluorescein), and an antimonocyte antibody such as phycoerythrin-conjugated anti-CD14. Cytofluorographic analysis of the cell population is assessed by fluorescence-activated cell sorters. $HLA\text{-}DR^+$ $CD14^-$ cells represent the dendritic cell population. In general, dendritic cells are readily distinguished from other PBML on the basis of their high

Example 3

Loading Disease-Associated Antigens onto Dendritic Cells

In the preferred embodiments, dendritic cells are introduced into the extracorporeal blood stream at any stage during photopheresis. More preferably, the dendritic cells are acid-eluted or agent treated as discussed above prior to introduction to the extracorporeal blood stream. Acid elution or agent treatment (such as using 8-MOP/UVA) of the dendritic cells induces release by the cells of peptides that may have become associated with their MHC Class I molecules during cell isolation and/or culture. Accordingly, in the most preferred embodiments, the acid-eluted dendritic cells are introduced to the extracorporeal blood stream at a temperature less than physiological temperature (between about 22°C and 27°C) to enhance dendritic cell empty MHC stability and minimize enzymatic antigenic peptide degradation.

Introduction of the dendritic cells into the extracorporeal blood stream is accomplished using standard injection ports (i.e., ports on the intravenous tubing sets) known to those of ordinary skill in the art. Preferably, between $1x10^6$ and $10x10^6$ dendritic cells constitute a single dose for injection into the subject. However, as discussed above, a number of dendritic cells to support multiple doses (e.g., 100-fold the number of cells for a single dose) can be introduced into the extracorporeal blood stream to prepare a stock preparation of antigen-loaded dendritic cells for storage and subsequent booster immunizations. In the preferred embodiments, this preparation of antigen-loaded dendritic cells is stored in aliquots containing a single dose for re-injection into the subject. In general, in a single dose of antigen-loaded dendritic cells, 300 to 300,000 MHC sites per cell are occupied by disease-associated peptides. More preferably, between about 1000 and 200,000 MHC site per cell are occupied by disease-associated peptides.

Following injection of the dendritic cells, the disease-associated antigens which are present in the extracorporeal blood stream following their release from the disease effector cells are loaded onto the MHC sites of the exogenously added dendritic cells. The photopheresis process steps (e.g., centrifugation) provide sufficient agitation and mixing of the dendritic cells and the disease effector cells from which the antigens are

GM-CSF (preferably at 30 U/ml). Preferably, the dendritic cells are subjected to acid elution (as described in Example 2, above), washed and placed in an appropriate loading medium at less than physiological temperature prior to contacting the dendritic cells with the disease-associated antigens. Loading the dendritic cells at a temperature less than physiological temperature enhances empty MHC stability and thereby maximizes the number of MHC sites available for association with the disease-associated antigens. Reduced temperature loading also minimizes the likelihood of enzymatic digestion of the released peptide antigens.

The disease-associated antigen preparation of the invention is added to the dendritic cell cultures and the cultures are incubated with the antigen for several hours or for sufficient time to allow the dendritic cells to present the antigen in a form which is recognized by T cells. Preferably, the cultures are incubated at a temperature less than physiological temperature to maximize the number of empty MHC sites available for antigen loading. Following loading of the disease-associated antigens into the dendritic cell MHC sites, the cells are collected from the culture, washed extensively and are used to immunize the subject. Of course, a known control antigen can be included in the preparation as a control and a portion of the collected cells can be devoted to a quality control assay to determine (1) the viability of the dendritic cells and/or (2) the functional activity of the antigen-loaded dendritic cells with respect to their ability to induce an antigen-specific immune response *in vitro* (e.g., a cytotoxic T cell assay) or *in vivo*. For example, the peptide-loaded dendritic cells can be injected subcutaneously into a mouse in an amount sufficient to induce an immune response to the known control antigen to estimate the efficiency of antigen loading for the tested cells. In the preferred embodiments, the antigen-loaded dendritic cells are irradiated (3000 rads gamma irradiation) before injection (preferably, i.v., or i.d. injection). More preferably, the antigen-loaded dendritic cells are coadministered with one or more cytokines (e.g., GM-CSF, IL-12, IL-4) to further enhance a specific immune response to the disease-associated antigen. (See, e.g., Zitvogel *et al.*, *J Exp Med* 184:87-97 (1996) which reports that co-administration of peptide-pulsed dendritic cells with low doses of IL-12 may favor the priming of tumor-specific T cells).

dendritic cells to prevent cell proliferation when the cells are reintroduced into the subject.

Example 4

Demonstrated Efficacy of Photopheresis/Dendritic Cell Treatment

To demostrate the effectiveness of combining extracorporeal treatment of disease effector cells and the addition of dendritic cells, murine 2B4.11 tumorigenic T cells were treated as described above using 8-MOP and UVA. The 2B4.11 tumor cells were derived by hybridizing or combining two original cell types: normal AKR mouse T cells with a BW5147 mouse malignant T cells. The AKR parental cell provides specific antigens, including a T cell receptor, which can apparently serve as a tumor specific antigen, to be targeted by an induced anti-tumor immunologic reaction. The BW5147 parental cell contribution permits the 2B4.11 cells to act like a cancer cell, dividing without check until they kill the animal.

Following 8-MOP/UVA treatment, dendritic cells were added to the treated cell mixture.

Two groups of 5 test mice were vaccinated with 5 million 2B4.11 cells that had been inactivated with 8-MOP/UVA treatment and mixed with dendritic cells (DPAC). The 8-MOP/UVA irradiated tumorigenic cells were shaken overnight with 200,000 dendritic cells (a 25 to 1 ratio), to maximize cell-to-cell contact between the DAPCs and the 2B4.11 cells. One group of cells (irradiated 2B4.11 plus DAPCs) was incubated at 23° C, to maximize stability of empty class I MHC molecules on the DAPCs. The other group of cells was incubated at 37° C, to maximize normal cellular metabolism. The combined irradiated 2B4.11/DAPC cell mix was injected into the test mice one week prior to challenging the animals with viable, tumorigenic 2B4.11 cells.

In Figure 1, reading from left to right, tumor growth is first depicted in five control mice, which received only skin injections of tumor cells on day 0 and no anti-tumor vaccination. All of these mice had visible tumors by day 8, which progressively grew until day 21, the last day of observation in these experiments.

substantial, but does not lead to the specific immunologic memory which follows vaccination with the 2B4.11/DAPC mix shown in the Figure 1. For example, although not shown in these graphs, if a second 2B4.11 tumor is administered after the resolution of the tumors, immunoprotection is only seen in the group getting the combination vaccination of 2B4.11 mixed with DAPCs.

Example 6

Demonstrated Increase in MHC Expression Following Photopheresis

Figure 3 shows the impact of 8-MOP/UVA on Class I expression. Mean fluorescence channel quantifies the amount of Class I protein on the cell surface, as identified with a fluorescent antibody against the Class I protein. Normally, at least 200,000 Class I molecules are displayed, as shown by a value of 2.4 in the control cell population. After exposure to 8-MOP/UVA (1 joule per cm2 UVA and 200 ng/ml of 8-MOP) and overnight incubation, Class I expression doubles. Since this massive increase in Class I expression is prevented by emetine, it results from new protein synthesis.

It should be understood that the preceding is merely a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. All references, patents and patent publications that are identified in this application are incorporated in their entirety herein by reference.

5. The method of claim 4, wherein the photoactivatable agent is a psoralen.

6. The method of claim 1, wherein the diseased subject has a disease that is mediated by a disease effector cell selected from the group consisting of a T cell, a B cell, a virally infected disease effector cell, a bacterially infected disease effector cell a protozoally infected disease effector cell, and a solid tumor cell.

7. The method of claim 1, wherein the disease is selected from the group consisting of a leukemia, a lymphoma, solid tumors, an autoimmune disease, graft v. host disease, transplanted tissue rejection, a viral infection that is mediated by virally infected disease effector cells, a bacterial infection that is mediated by bacterially infected disease effector cells and a protozoan infection that is mediated by protozoally infected disease effector cells.

8. The method of claim 1, wherein the disease-associated antigen is a peptide that is bound to a protein selected from the group consisting of an MHC Class I protein, an MHC Class II protein and a heat shock protein that is capable of transporting peptide to or from an MHC site.

9. The method of claim 1, wherein the dendritic cells are introduced at a stage of extracorporeal treatment selected from the group consisting of stages consisting of (1) blood collection; (2) disease effector cell isolation; (3) agent treatment; and (4) disease effector cell pooling.

10. The method of claim 1, wherein said dendritic cells and said disease effector cells are separated from each other during the contact step by a membrane that allows antigens and cytokines to pass through but does not allow the cells to pass through.

18. The process of claim 17, wherein the disease effector cells are selected from the group consisting of malignant T cells, malignant B cells, T cells which mediate an autoimmune response, B cells which mediate an autoimmune response, T cells which mediate transplanted tissue rejection, B cells which mediate transplanted tissue rejection, solid tumor cells, virally infected disease effector cells which express on their surface viral proteins, bacterially infected disease effector cells which express on their surface bacterial proteins and protozoally infected disease effector cells which express on their surface protozoan proteins.

19. The process of claim 18, wherein the disease effector cells are isolated from peripheral blood prior to acidification.

20. The process of claim 19, further comprising the step of:

(c) dividing the product into aliquots, each aliquot containing an amount of product sufficient for mixing with a single dose of antigen presenting cells to enhance an immune system response.

21. A product for enhancing an immune response, the product produced by the process of claim 19.

22. In a method for photopheresis, the improvement which comprises increasing at least one of the number and activation state of dendritic cells in a subject via the steps of:

(1) administering a sufficient dosage of GM-CSF to the subject; and

(2) subjecting the subject to photopheresis,

wherein the sufficient dosage of GM-CSF is an amount and frequency of administration of GM-CSF that is sufficient to increase at least one of the number and activation state of the dendritic cells in the subject.

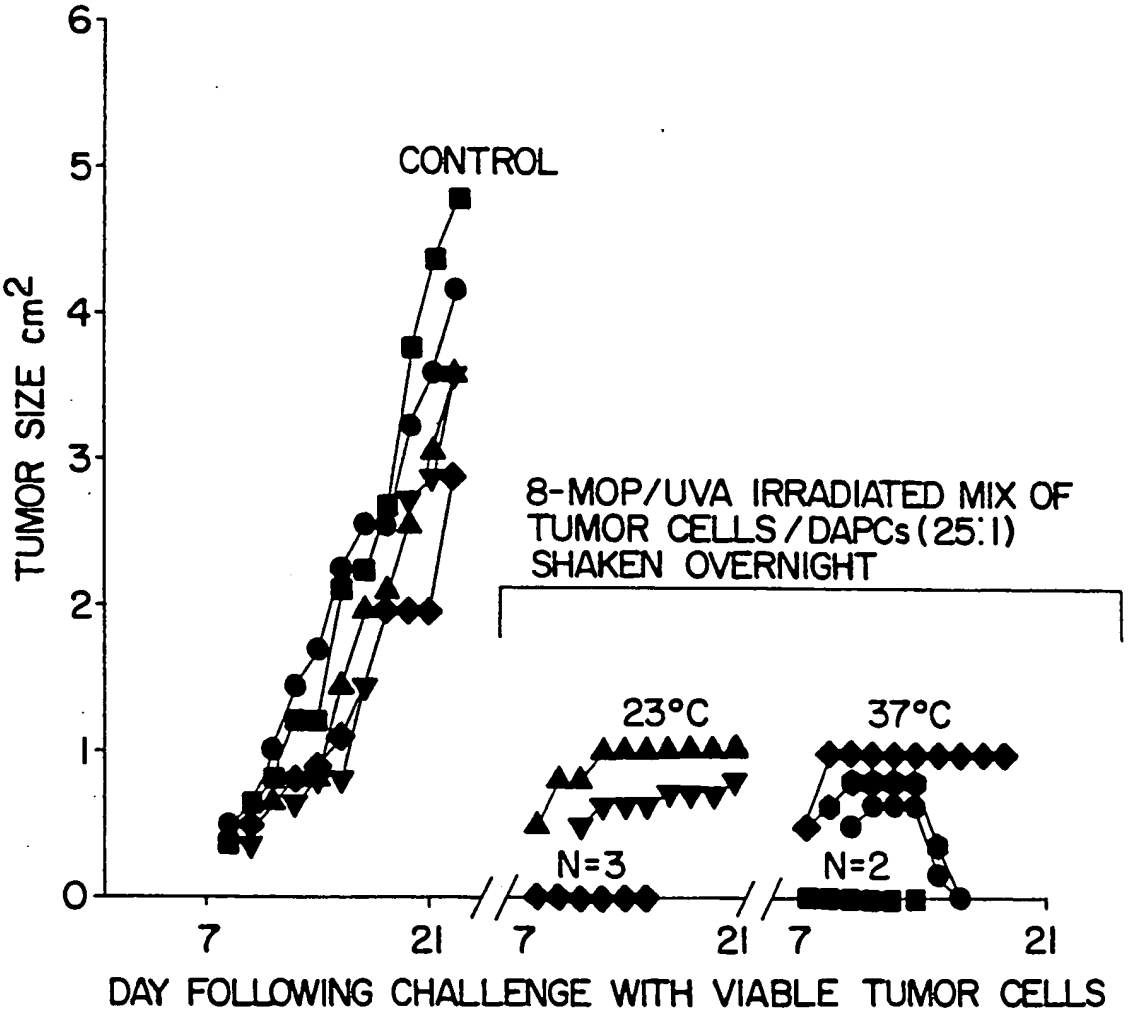
TUMOR SIZE cm2
6
5
4
3
2
1
0
CONTROL
8-MOP/UVA IRRADIATED MIX OF
TUMOR CELLS / DAPCs (25:1)
SHAKEN OVERNIGHT
23°C
37°C
N=3
N=2
7
21
7
21
7
21
DAY FOLLOWING CHALLENGE WITH VIABLE TUMOR CELLS

FIG. 1

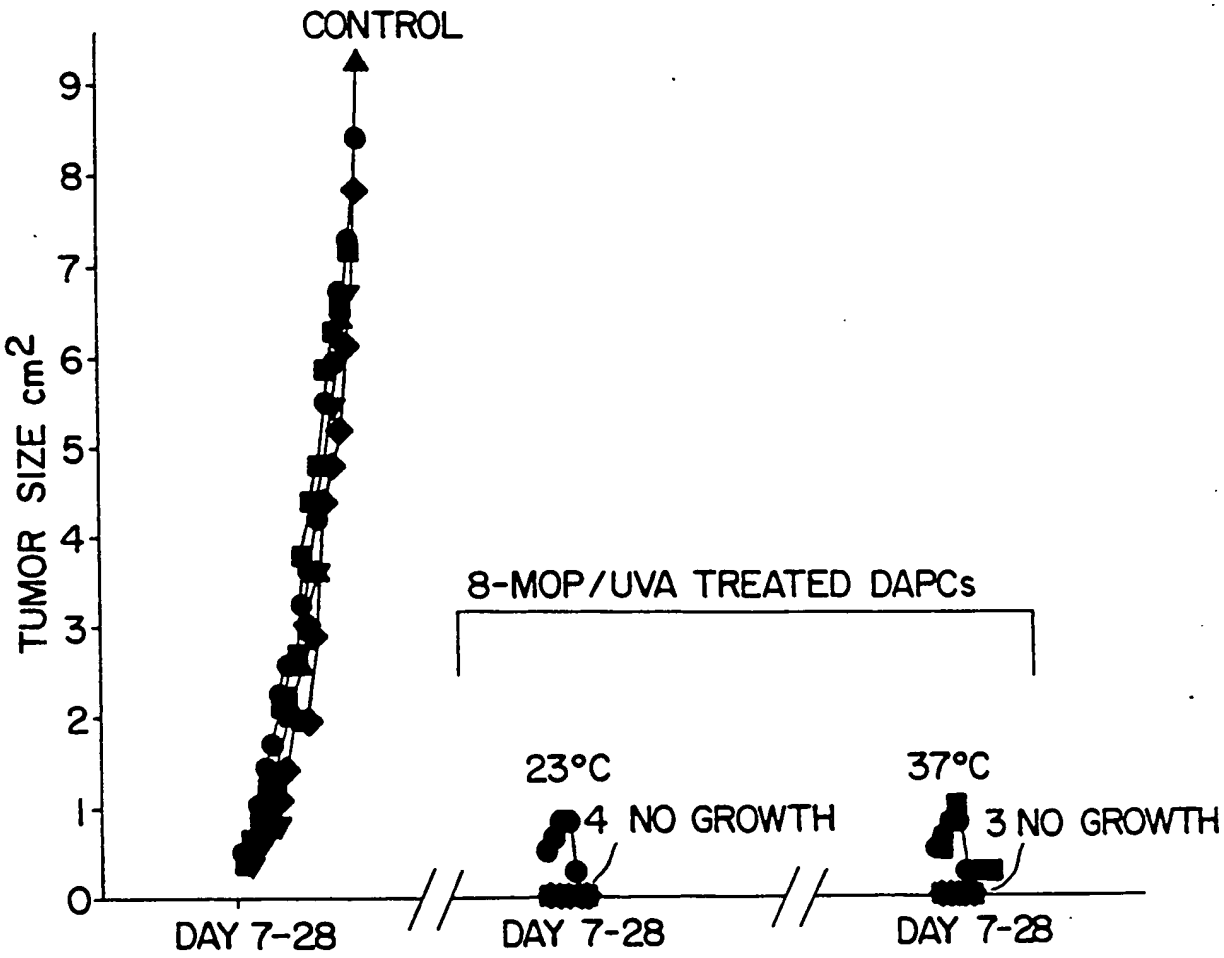
CONTROL
TUMOR SIZE cm2
0
1
2
3
4
5
6
7
8
9
8-MOP/UVA TREATED DAPCs
23°C
4 NO GROWTH
37°C
3 NO GROWTH
DAY 7-28
DAY 7-28
DAY 7-28

FIG. 2

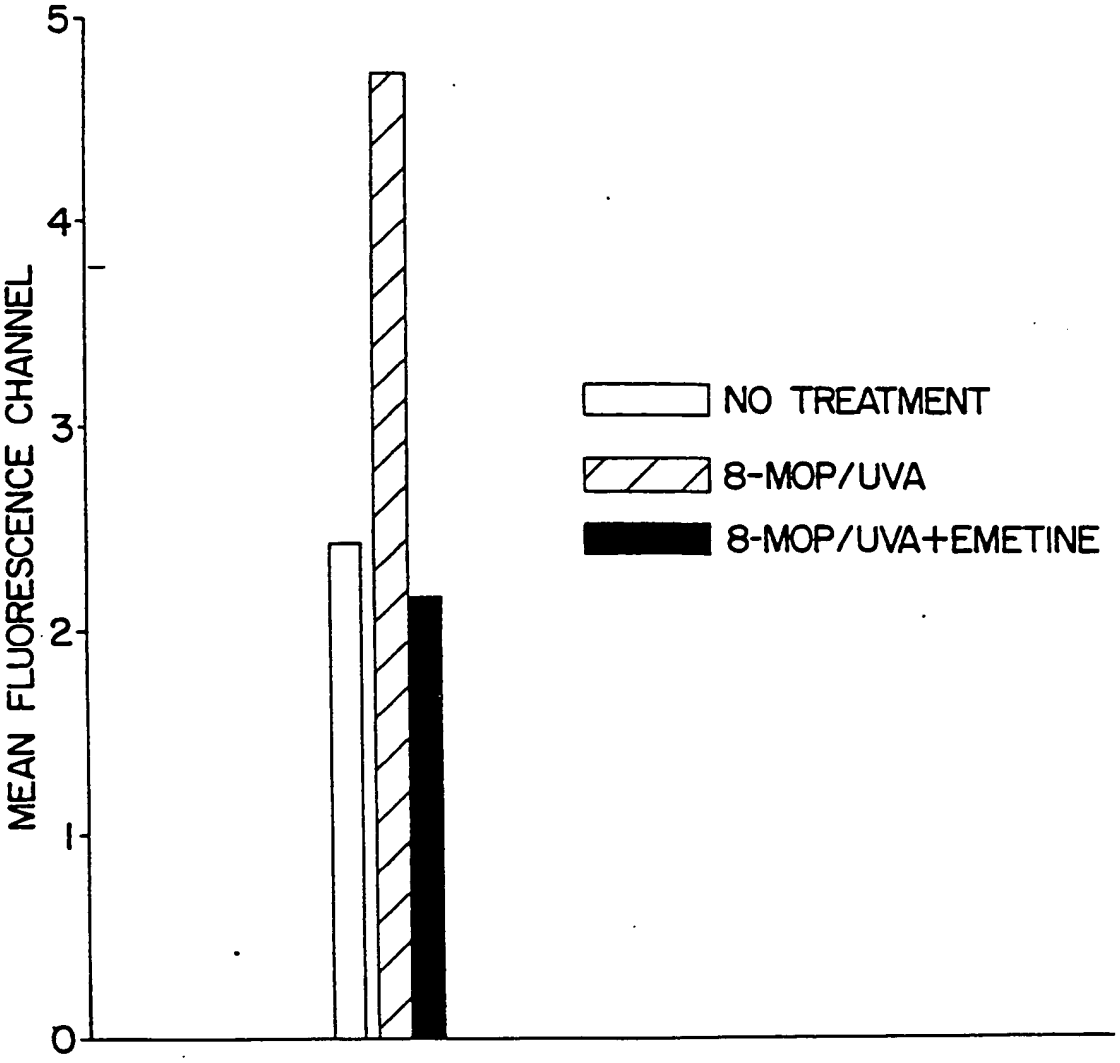
MEAN FLUORESCENCE CHANNEL
0
1
2
3
4
5
NO TREATMENT
8-MOP/UVA
8-MOP/UVA+EMETINE

FIG. 3